

Canine gastrointestinal microbiome in health and disease



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Dr. Simpson qualified from the Royal (Dick) School of Veterinary Studies, University of Edinburgh, in 1984 before gaining his PhD from the University of Leicester. He then lectured at the Royal Veterinary College in London before moving to Cornell University in 1995; he was appointed Professor of Medicine at Cornell in 2007. His main interest is the interplay between bacteria and host that leads to chronic inflammatory disease and cancer, aiming to effectively translate laboratory-based studies into better disease detection, therapy and ultimately prophylaxis for both animals and humans.

KEY POINTS

- Advances in microbiology have revealed a much more abundant, diverse, and complex gastrointestinal microbiota than was previously appreciated using culture-based methods.
- Contemporary culture-independent microbiology, based on the detection of molecular signatures of bacteria such as 16S and 23S rRNA genes, enables in-depth evaluation of the presence and localization of bacteria in the gut.
- The intestinal microbiota has a key role in maintaining health and immunity.
- Dysbiosis, or imbalances in the intestinal microbiota, are increasingly associated with inflammatory bowel disease (IBD).
- Culture-independent methods have enabled the discovery of mucosally invasive bacteria in dogs with granulomatous colitis.
- A combination of dysbiosis and host susceptibility may influence the response to antibiotics seen in dogs with antibiotic-responsive enteropathies.
- Elucidating the factors that shape the intestinal microbiome will provide novel opportunities for prophylaxis and therapeutic intervention.

■ Introduction

The intestinal microbiota is defined as the aggregate of all living micro-organisms (bacteria, fungi, protozoa, and viruses) that inhabit the gastrointestinal (GI) tract. The word microflora is often used in older textbooks, but microbiota (from the word *bios* in ancient Greek meaning "life") is the more appropriate term.

Until a few years ago, culture was the principal method used to identify bacteria inhabiting the canine GI tract, and this technique still yields useful result when employed for detection of specific enteropathogens (e.g. *Salmonella*, *Campylobacter jejuni*). However, it is now well recognized that the vast majority of intestinal microbes present in the GI tract remain undetected using culture-based methods (1). A new molecular method, known as 16S rRNA sequencing, allows bacteria to be identified in a much more reliable way using a culture-independent approach. Bacterial DNA is extracted from an intestinal sample and the 16S rRNA gene is amplified and processed via PCR using a high-throughput sequencer, allowing a more comprehensive identification of the bacteria present in the sample (**Figure 1**). Such molecular studies have revealed that the canine GI tract is home to a highly complex microbial ecosystem, referred to as the intestinal microbiome, consisting of several hundred different bacterial genera and probably more than a thousand bacterial

phylotypes (2). It has been estimated that the intestinal microbiome consists of approximately 10 times more microbial cells (10^{12} - 10^{14}) than the number of host cells, and the microbial gene pool is 100-fold larger compared to the host gene pool. It is emerging that this highly complex microbial ecosystem plays a crucial role in regulation of host health and immunity, as demonstrated in various studies in humans, animal models, and (most recently) dogs and cats (1).

The microbial metabolites produced by the resident microbiome are thought to be one of the most important driving forces behind the co-evolution of GI microbiota with their host (**Table 1**). Gut microbes benefit the host in several ways; they act as a defensive barrier against transient pathogens, aid in nutrient breakdown and energy harvest from the diet, provide nutritional metabolites for enterocytes, and play a crucial role in the regulation of the host immune system. In contrast, various GI disorders have been associated with alterations in the composition of the intestinal microbiota (dysbiosis) in dogs, such as chronic enteropathies and granulomatous colitis in boxer dogs.

■ GI microbiota of healthy dogs

As noted above, molecular-phylogenetic analysis of the bacterial 16S rRNA gene has created a more detailed inventory of the bacterial groups present in the GI tract and has revolutionized our understanding of the complex gut ecology. Aerobic bacteria occur in relatively higher proportions in the small intestine, while the large intestine harbors almost exclusively anaerobic or facultative anaerobic bacteria. The bacterial phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, and *Fusobacteria* constitute approximately 99% of all gut microbiota in dogs (2,3). Those phyla can be subdivided phylogenetically into several bacterial families and genera (**Figure 1**). *Helicobacter spp.* are the major group found in the canine stomach; the small intestine harbors predominantly *Clostridia*, *Lactobacillales*, and *Proteobacteria*; and *Clostridiales*, *Bacteroides*, *Prevotella*, and *Fusobacteria* dominate in the large intestine. The phylum *Firmicutes* comprises many phylogenetically distinct bacterial groups, the so-called *Clostridium* clusters. These groups (e.g. *Ruminococcus spp.*, *Faecalibacterium spp.*, *Dorea spp.*), together with *Bacteroidetes* and *Actinobacteria* (*Bifidobacterium spp.*) are believed to be important producers of metabolites (e.g. short-chain fatty acids, indole) that have a direct beneficial impact on host health (**Table 1**).

Of special note is that each animal harbors a very unique and individual microbial profile. These differences in bacterial composition between individual animals may explain,

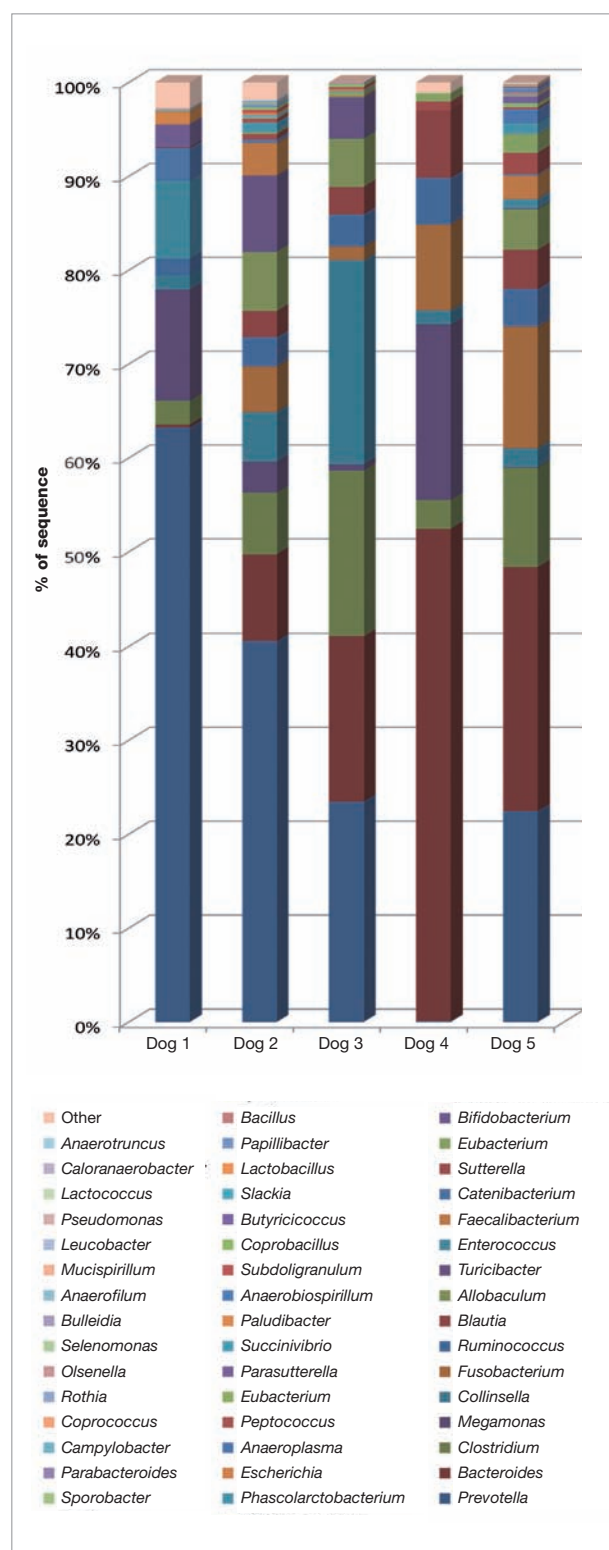


Figure 1. Predominant bacterial genera observed in fecal samples of five healthy dogs. Data was derived from high-throughput sequencing of the 16S rRNA gene (22). Note how the type and number of bacterial groups vary between individual dogs.

Table 1. Microbial-derived metabolites in the GI tract.

Metabolic end products	Metabolic activities of intestinal microbiota	Effect on host health
Propionate, acetate, butyrate	Carbohydrate fermentation	Anti-inflammatory, energy source of enterocytes, regulation of intestinal motility, amelioration of leaky gut barrier
Retinoic acid (Vitamin A derivate)	Vitamin synthesis	Important for generation of peripheral regulatory T-cells
Vitamin K2, B12, biotin, folate	Vitamin synthesis	Important co-factors for various metabolic pathways
Ceramide	Induces degradation of sphingomyelin via alkaline sphingomyelinase	Significant role in apoptosis and in the prevention of intestinal epithelial dysplasia and tumorigenesis
Indole	Degradation of the amino acid tryptophan	Increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation
Secondary bile acids (cholate/deoxycholate)	Deconjugation/dehydroxylation of bile acids	Intestinal fat absorption
Taurine	Bacterial deconjugation of bile acids	Facilitates fat absorption from the GI tract, important for liver metabolism
Oxalyl CoA decarboxylase	Degradation of oxalate through oxalyl CoA decarboxylase	Decreases in oxalate degrading enzyme associated with increased risk for calcium oxalate urolithiasis
Ammonia	Decarboxylation, deamination of amino acids	Increases associated with encephalopathy
D-lactate	Carbohydrate fermentation	Increases associated with encephalopathy

in part, the highly individualized response observed to therapeutic approaches that are designed to modulate intestinal microbiota.

In addition to knowing the inventory of GI bacteria it is important to consider their distribution within the intestinal lumen and the mucosa. The regional and spatial distribution of bacteria within the GI tract can be analyzed at the molecular level using fluorescence *in situ* hybridization (FISH) assays. The normal canine stomach typically harbors abundant *Helicobacter* species that colonize the superficial mucosa, gastric glands, and parietal cells (**Figure 2**) (4,5). The mucosa of the large intestine is also home to a large number of mucosa-associated bacteria including *Helicobacter* species, whereas very few bacteria are seen in association with the small intestinal mucosa (**Figure 2**). Apart from the stomach, where intra-mucosal *Helicobacter* are frequently visualized, mucosally invasive bacteria are absent in the healthy small and large intestines.

While the recent literature has started to provide a solid overview of the composition and spatial distribution of the

canine GI microbiota, further studies are needed to unravel disease associations and functional changes in health and disease.

■ Microbiota in immunity and health

A balanced microbial ecosystem is crucial for optimal health. The physiologic microbiota provides stimuli for the immune system, helps in the defense against invading enteropathogens, and affords nutritional benefits to the host (**Table 1**). The resident microbiota is important in the development of the physiological gut structure. For example, germ-free animals exhibit an altered mucosal architecture (e.g. decreased number of lymphoid follicles, smaller villi). The microbiome in early life is crucial for establishing oral tolerance in order to prevent onset of inappropriate immune responses against bacterial and food antigens, which have been associated with chronic GI inflammation.

There is a constant “cross-talk” between intestinal bacteria and the host immune system that is believed to be mediated through a combination of microbial metabolites and surface molecules that activate innate immune receptors

(e.g. Toll-like receptors or TLRs) in the intestinal lining. The resident intestinal microbiota is also a crucial part of the intestinal barrier system that protects the host from invading pathogens as well as deleterious microbial products (e.g. endotoxins). Examples include the competition for nutrients, for mucosal adhesion sites, and the creation of a physiologically restrictive environment for non-resident bacterial species (e.g. secretion of antimicrobials, alterations in the gut pH, hydrogen sulfide production).

The canine colon harbors almost exclusively anaerobic or facultative anaerobic bacteria. As shown in **Figure 1**, predominant colonic bacterial groups are part of the *Prevotella/Bacteroides* group and the *Clostridium* clusters (e.g. *Lachnospiraceae*, *Ruminococcaceae*, *Faecalibacterium* spp.) (2). Some of the major nutrient sources of bacteria are complex carbohydrates, including intestinal mucus, starch and dietary fiber such as pectin and inulin. The fermentation of these substrates results mainly in the production of short-chain fatty acids (SCFA) - such as acetate, propionate, and butyrate - and other metabolites which are important energy sources for the host. SCFA are important growth factors for intestinal epithelial cells; they have immunomodulatory properties, they may inhibit the overgrowth of pathogens via modulation of colonic pH, and they also influence intestinal motility (6). Butyrate protects against colitis through reduction of oxidative damage to DNA and through induction of apoptosis of cells with DNA damage. Acetate has been shown to beneficially modulate intestinal permeability, thereby decreasing the systemic translocation of gut microbiota-derived endotoxins (6). Furthermore, recent metabolomics studies suggest that the different members of the intestinal microbiota produce various other immunomodulatory metabolites (e.g. histamine, indole). For example, *in vitro* studies have shown that microbial-derived indole decreases IL-8 expression, induces expression of mucin genes, and also increases gene expression that strengthens tight junction resistance (7).

■ **Microbiota in dogs with GI disease**

As detailed above, the resident microbiota is an important driver of host immunity. It is anticipated that changes in the composition of the microbiota (dysbiosis) will have significant impact on host health. These effects can manifest themselves in the GI tract, but because of the importance of the microbiota on the gut-associated lymphoid tissue (GALT) the effects of intestinal dysbiosis can have far-reaching impacts on extra-intestinal organ systems (**Table 2**).

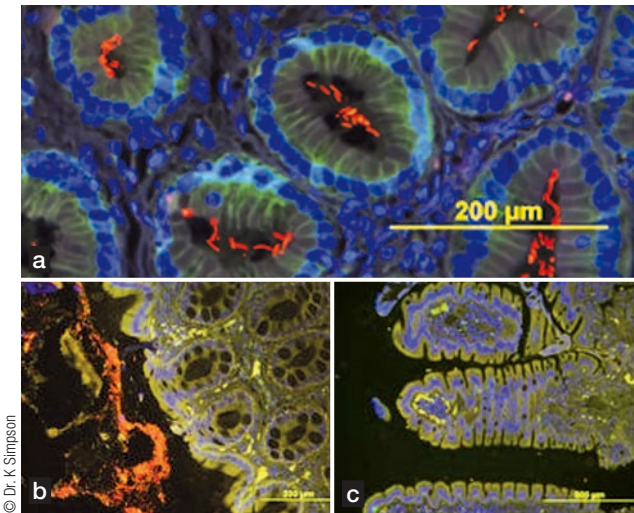


Figure 2. Spatial distribution of bacteria throughout the healthy GI tract can be examined via FISH analysis, whereby bacteria stain red and nuclei stain blue. The normal canine stomach frequently harbors abundant *Helicobacter* species that colonize the superficial mucosa, gastric glands, and parietal cells (a). The mucosa of the large intestine is also home to a large number of mucosa-associated bacteria (b). Very few mucosal bacteria are found in the small intestine (c).

Table 2. Disorders associated with changes in the intestinal microbiome.

Disorder	Affected species
Acute hemorrhagic diarrhea	Dogs
Atopic dermatitis	Humans, mouse models, dogs
Autism	Humans
Calcium oxalate (CaOx) urolithiasis	Dogs
Diabetes mellitus type II	Humans, rodent models
Inflammatory bowel disease	Humans, rodent models, dogs, cats
Irritable bowel syndrome	Humans
Metabolic syndrome	Rodent models
Obesity	Mouse models
Stress diarrhea	Humans, rodent models, dogs
Stress, anxiety, depression-related behavior	Mouse models

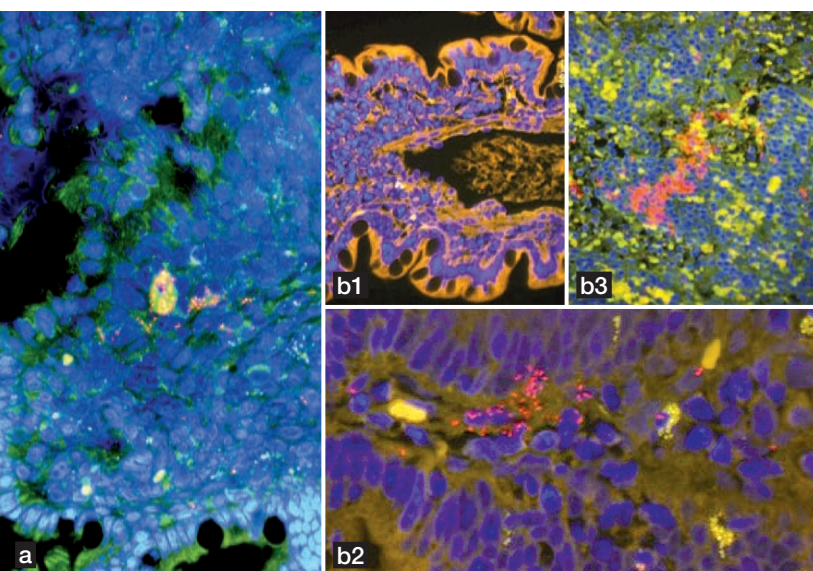


Figure 3. *In situ* detection of invasive bacteria associated with granulomatous colitis and ileitis:

a. Invasive *E. coli* in the colon of a boxer with granulomatous colitis.

b. The presence of bacteria (red) in a dilated lacteal (b1), villus (b2) and mesenteric lymph node (b3) of a 7-year-old bichon frise with pyogranulomatous ileitis. Histochemical staining of intestine and regional lymph node with periodic acid-Schiff (PAS), acid fast and Gram stains was negative.

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Enteropathies associated with mucosally invasive bacteria

The application of 16S rRNA gene sequence-based analysis in combination with FISH assays has enabled the discovery of invasive bacteria in the colonic mucosa of boxers with granulomatous colitis (8). Comparison of 16S rRNA gene libraries before and after antibiotic-induced remission revealed significant enrichment in gram-negative sequences with highest similarity to *E. coli* and *Shigella*. *In situ* analysis with FISH probes against *E. coli* showed multifocal clusters of invasive bacteria within macrophages. Subsequent studies have shown that granulomatous colitis in French bulldogs is also associated with mucosally invasive *E. coli*. Eradication of invasive *E. coli* in boxer dogs and French bulldogs with granulomatous colitis correlates with remission from disease, inferring a causal relationship (9). The types of *E. coli* isolated from boxer dogs resemble those associated with Crohn's disease in people (8,10). IBD across species is increasingly considered to involve interplay between the intestinal microenvironment (principally bacteria and dietary constituents), host genetic susceptibility, the immune system, and environmental "triggers" of intestinal inflammation (10,11). The predisposition of boxer dogs and French bulldogs to *E. coli*-associated granulomatous

colitis suggests they may harbor a genetic defect or defects that impairs their ability to kill invasive *E. coli*. Invasive bacteria can also be involved in granulomatous and neutrophilic IBD in other breeds and other regions of the gut (**Figure 3**). Given the increasingly recognized association of granulomatous and neutrophilic IBD with infectious agents it seems prudent to perform specialized testing for bacteria and fungi before considering any form of immunosuppressive treatment.

Antibiotic-responsive enteropathies without mucosally invasive bacteria

Historically, dogs with signs of chronic GI disease that lacked an intestinal obstruction and resolved with antimicrobial therapy were diagnosed as having "idiopathic small intestinal bacterial overgrowth" or SIBO (12,13). However, after it was shown that total bacterial numbers in these dogs were similar to healthy dogs and dogs with food or steroid-responsive enteropathies or EPI, (14,15) the term "antibiotic-responsive enteropathy" was coined to describe this syndrome. Certain breeds, such as the German shepherd dog (GSD) appear predisposed to antibiotic-responsive enteropathies (13). Histopathological findings in GSD and other dogs with antibiotic-responsive enteropathies have frequently been reported as normal or showing mild lymphocytic-plasmacytic IBD.

In the absence of florid inflammation or invasive bacteria the reason for the response to antibiotics has been unclear. However, recent studies in dogs with chronic enteropathies have implicated abnormalities in the innate immune system that may amplify inflammatory responses to the resident microbiota. Toll-like receptors (TLRs) are membrane-spanning receptors that play a key role in both the immune system and the digestive tract. Polymorphisms in TLR5 (which recognizes flagellin, a protein that forms the filament in bacterial flagellae) and increased TLR4 and decreased TLR5 expression have been demonstrated in GSDs when compared to healthy greyhounds (**Figure 4**) (16,17). In addition, four non-synonymous single nucleotide polymorphisms (SNPs) were identified in the canine NOD2 gene (17) and this was significantly more frequently found in IBD dogs than in controls. These results were also mirrored in non-GSD breeds (18).

The recent demonstration that polymorphisms in TLR5 confer hyperresponsiveness to flagellin suggests that the antibiotic response observed in GSDs is a consequence of reduced intraluminal flagellin (19). Culture-independent analysis of the intestinal microbiota of GSDs with chronic enteropathies indicates increased abundances of

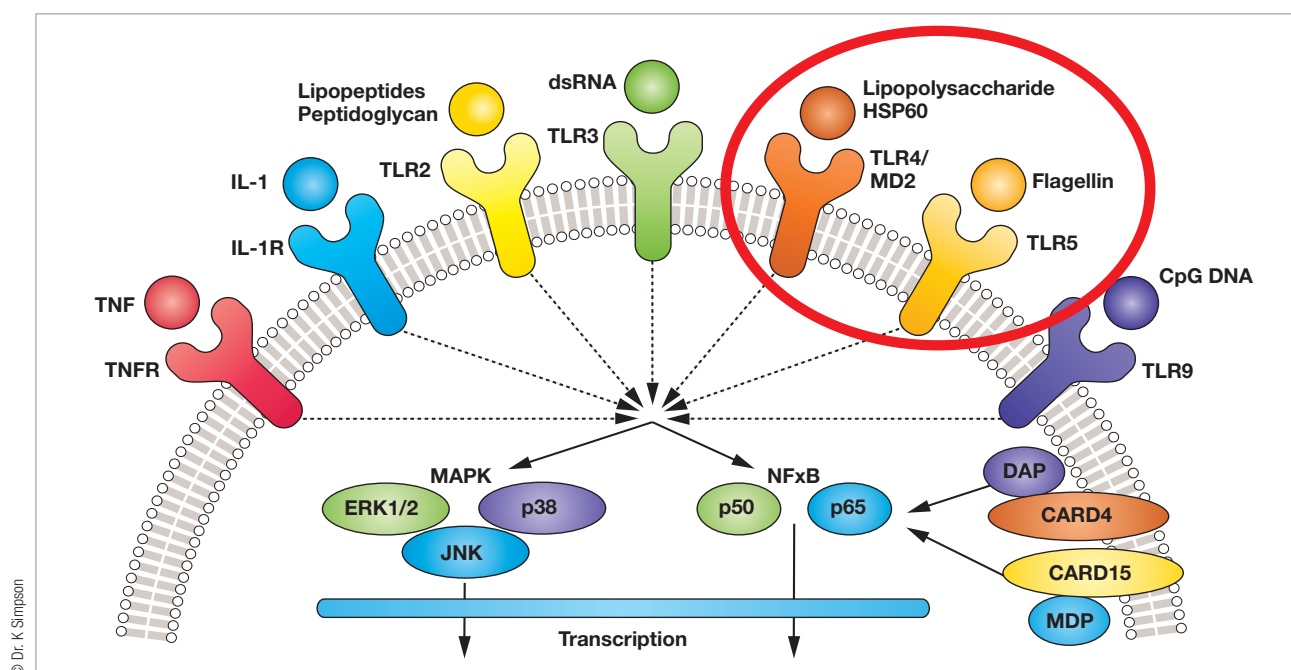


Figure 4. Host susceptibility and the enteric microbiota interact to promote intestinal inflammation. Toll-like receptors (TLRs) are membrane-spanning receptors that play a key role in both the immune system and the digestive tract, recognizing foreign proteins and activating immune cell responses. Polymorphisms in the TLR4 and TLR5 gene are significantly associated with IBD in GSDs; bacteria signaling through aberrant TLR5 has been shown to result in hyper-responsiveness to flagellin.

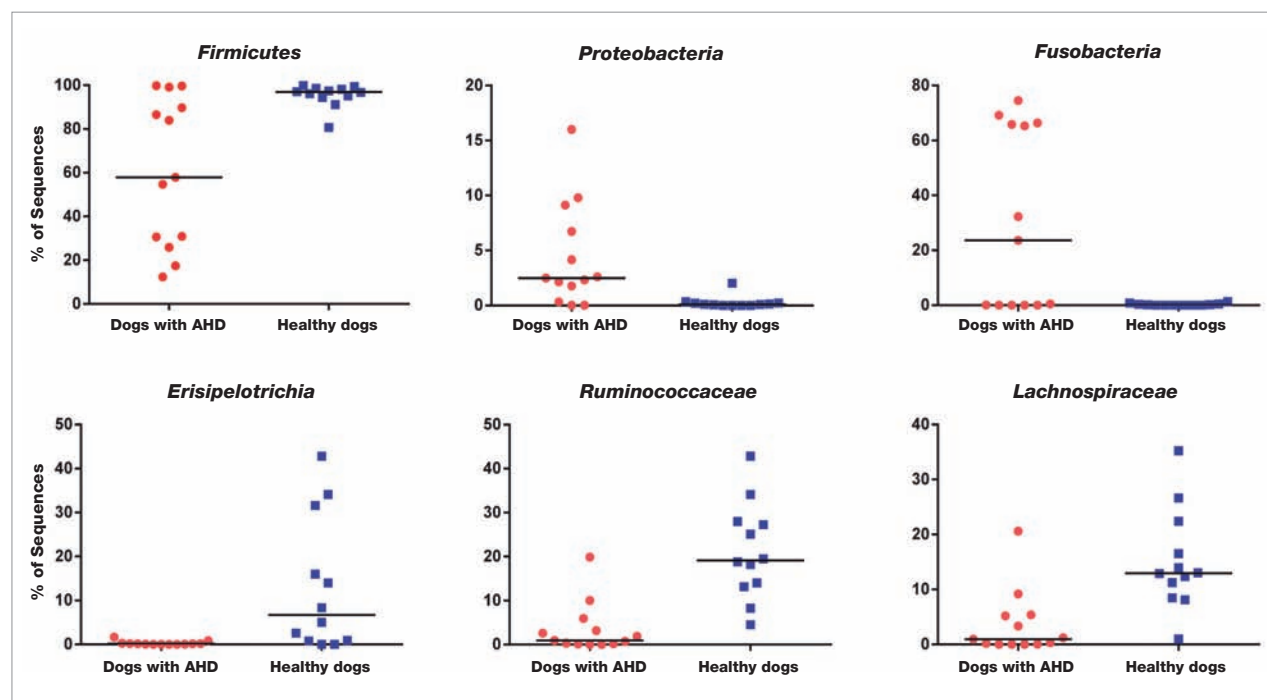


Figure 5. Differences in the major bacterial groups in fecal samples of healthy dogs and dogs with acute hemorrhagic diarrhea (AHD). Data was derived from high-throughput sequencing of the 16S rRNA gene (22). The results indicate a pronounced dysbiosis in dogs with diarrhea, as the majority of the normal microbiota is depleted in disease. These changes are most likely accompanied by reductions of beneficial microbial-derived metabolites, although to date the extent of the metabolic consequences has not been examined in depth.

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Lactobacillales compared to healthy greyhounds (16). The relationship between dysbiosis, clinical disease, and enhanced inflammatory responses remains to be clarified.

Positive clinical responses to the macrolide antibiotic tylosin have also been consistently reported in subsets of dogs with chronic enteropathies (20). Recently, the small intestinal microbiota has been analyzed in dogs following tylosin administration, providing potential clues on the effect of this antibiotic on intestinal microbes (21).

Conclusion

Taken as a whole, the microbial alterations documented in dogs with chronic GI disease are comparable to those observed across species where a shift in the microbiome from gram-positive *Firmicutes* (e.g. *Clostridiales*) to gram-negative bacteria, predominantly *Proteobacteria* (including *Enterobacteriaceae*), correlates with intestinal inflammation (10,22-24). This depletion of commensal groups may impair the ability of the host to down-regulate the aberrant intestinal immune response, as several of these

bacterial groups secrete metabolites that have direct anti-inflammatory properties (24). However, at this time the relationship between microbial alterations and inflammation is not well understood. Is dysbiosis a cause or a consequence of inflammation? Acute enteritis in dogs is associated with dysbiosis, especially depletions of bacterial groups that are important producers of SCFA and other microbial metabolites (**Figure 5**) (22), suggesting that bacterial changes are a consequence of the inflammatory response, but they may influence inflammation in genetically susceptible hosts. Recent experimental studies have shown that acute inflammation, triggered by protozoan infection and NSAID administration, can induce dysbiosis that parallels the shifts observed in Crohn's disease, and that host genetics may impact the threshold and the magnitude of dysbiosis (25). Clearly we are only just beginning to unravel the complex interrelationships between the enteric microbiota, health and disease. Elucidating the factors that shape the intestinal microbiome will provide novel opportunities for prophylaxis and therapeutic intervention in dogs with IBD.

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